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Role of T-Bet in Production of Immunoglobulin Isotypes in an Influenza Setting

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ROLE OF T-BET IN PRODUCTION OF IMMUNOGLOBULIN ISOTYPES IN AN INFLUENZA SETTING

by

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Abstract

Influenza is one of the most common diseases worldwide, yet the vaccines against influenza are only 35% effective at protecting against infection. Creating a more effective vaccine requires an understanding of the foundation and the factors that contribute to a strong and protective adaptive immune response. T-bet [*TBX21*] is a transcription factor that plays an instrumental role in the orchestration of the type 1 immune response, which is the specialized response used by the immune system for a cell-mediated response against intracellular pathogens, such as influenza. It has yet to be explored in an influenza setting on the role T-bet in the production of antibodies. The aim of this study is to understand T-bet's role in production of antibody isotypes and identify whether expression of T-bet is more important for antibody production in T cells or B cells. We expected T-bet knockout (KO) mice to have IgG_{2a} and that T-bet expression would be more important in T cells for antibody production. An enzyme-linked immunosorbent assay (ELISA) was used to measure the amount of virus-specific antibody in T-bet KO versus wild type (WT) mice infected with influenza. The results show that the T-bet KO and WT mice have relatively the same amount of IgG and IgG_1, but the T-bet KO have a significantly lower level of IgG_{2a}, confirming T-bet's importance for its production. To distinguish the importance of T-bet expression while T-bet expression in T cells was constant, a model was developed to allow us to control expression of T-bet in B cells. The results however were inconclusive, and the experiment will have to be repeated to make a firm conclusion on the roles of lymphocytes in the control of IgG isotypes. Overall, these results indicate that the manipulation of T-bet expression can be used as a vector to control IgG antibody levels, which holds potential for the improvement of vaccines.

Acknowledgements

The candidate thanks Dr. Kai McKinstry for his patience, support and for the opportunity to conduct research. The candidate would also thank Kunal Dhume for his assistance with data analysis and procedures. The candidate also thanks Dr. Mollie Jewett and Dr. Deborah Altomare for their support and advice as members of the Committee.

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BACKGROUND

Influenza and Vaccination

Influenza A virus (IAV), commonly called the flu, is one of the most common diseases globally, with nearly 5 million severe cases and 500,000 deaths per year [1]. With the virus constantly mutating, vaccines must be administered during each flu season. The vaccines are also inefficient: aside from the constant mutations, scientists must determine the strain that is causing the epidemic. Vaccine effectiveness for the past three years averages only 35% [2-5]. Understanding the immune response against IAV, specifically the isotypes of antibody being produced and the effectiveness of each, can help produce a more protective vaccine. Not only would this prevent millions of flu cases and deaths every year, in both developed and developing nations, but the money this would save in both healthcare and the business industries is significant. On an even larger scale, understanding the factors that result in and effect the production and efficacy of adaptive immunity has greater implications in the treatment of other diseases and autoimmune disorders.

Vaccination is based on the understanding that upon being infected by, and clearing a pathogen, the body will be less susceptible to reinfection by that same pathogen. The immunology behind this is the creation of memory cells by the adaptive immune system that are specific for the infecting pathogen, and upon reinfection, allow for an immune response that is faster and stronger than before, thereby offering protection against it [4]. Therefore, it is not surprising that creating a safe and effective vaccine is one of the best ways to mitigate infection by even the deadliest and virulent of pathogens.

IAV Variants

The variants of IAV are based on the different subtypes of hemagglutinin (HA) and neuraminidase (NA) that the virus displays. These glycoproteins have different functions: hemagglutinin allows the IAV to bind to the target cell and neuraminidase helps the virus spread [5]. The differences in these proteins is what allows for a heterosubtypic challenge, as the antibodies produced against one strain are not protective against another strain. The variants used in this experiment include PR8 (H1N1), A/Phil (H3N2), and A/Alaska (H3N2). Both PR8 and A/Phil are virulent strains of IAV whereas A/Alaska is a vaccination strain, which is important to note as this may lend translation of the results to humans, in terms of assessing how T-bet expression may impact vaccine efficacy.

Immune System

There are two types of defense systems used by the body for protection, innate and adaptive. In the case of a pathogen bypassing the innate immune system, the adaptive immune system, comprised of T cells, B cells, and antibodies, is activated to generate a more sustained and specific immune response. It is also important to note that the innate immune response is required for an adaptive response, as the innate response sets the stage for the adaptive immune system. T cells are lymphocytes that develop in the thymus, where they produce an antigen-specific T cell receptor (TCR) through gene rearrangement. They then travel through the blood and lymphatics in search of their specific antigen, which, upon binding, will allow them to mature and begin their various effector functions. There are two main types of T cells consisting of CD8+ cytotoxic T cells, which target and kill infected cells, and CD4+ T helper (Th) cells,

which facilitate and optimize the immune response by stimulating other immune cells [6]. Naïve CD4+ T cells differentiate into different subtypes, as a result of specific cytokines, each with a specialized role, generally: Th1 protects against intracellular pathogens, Th2 and Th17 defend against extracellular pathogens, T-follicular helper (Tfh) provides help to B cells, and periphery T regulatory (pTreg) prevents inflammation-mediated injury to tissue [7-11].

B cells are lymphocytes that develop in the bone marrow, and also have a specific B cell receptor (BCR) produced by gene rearrangement. Upon finding their specific antigen, B cells will differentiate into plasma cells and produce large soluble amounts of their BCR, referred to as antibodies. These antibodies will bind to their specific antigen and allow for neutralization, opsonization for phagocytosis, or direct killing via protein complement [9].

Immunoglobulin Isotypes

Immunoglobulins, more commonly referred to as antibodies, are proteins secreted by B cells which bind to a specific antigen. An antibody consists of a variable region (F_v), which is the antigen-binding portion, and the constant region (F_c), which determines the class of the antibody [12]. Each antibody isotype has a specific role in the immune response: IgG is the most abundant antibody, has a high affinity for its antigen, and is characteristic of a secondary immune response, IgM is a pentamer found during the primary immune response with a low affinity but high avidity, IgA is a monomer in blood or a dimer found in mucous secretions, IgE is a monomer that protects against parasites and plays a role in allergic reactions, IgD is found on B cells, but its function has yet to be determined. As previously mentioned, the initial antibody produced is IgM, but as the infection progresses, and upon the interaction of various signaling

molecules, the plasma cell will undergo class switching, where the F_c region in the gene will be recombined to express a different class with different effector functions [13].

IgG also has several subtypes, the most abundant of which are IgG1 and IgG_{2a}, which generally respond to proteins and polysaccharides respectively [14]. Since they each have specific functions, the relative amounts of each have often been used as markers of what type of immune response is occurring. IgG_1 is associated with type 2 immunity while IgG_{2a} is indicative of type 1 immunity. IgG_{2a} also has more F_c receptor-mediated reactions, especially with protein complement activation, while IgG_1 has weaker F_c interactions.

Antibodies in IAV

Since IAV is an intracellular pathogen, type 1 immunity is employed against it. The antibodies specific against IAV are primarily of the IgG isotype, but of greater interest are the subtypes in play. Being a type 1 immune response, we would expect IgG_{2a} to be more prevalent. An interesting follow-up to this assumption is that the literature has shown that higher levels of IgG_{2a} were associated with greater clearance of and protection against IAV [9]. These findings are significant in relation to T-bet because of T-bet's role in the class switching of B cells to the IgG_{2a} subtype. This is even more important in the context of producing a more effective vaccination against IAV.

Immune Response Regulation

Upon encountering a pathogen, the body can utilize a variety of immune responses for protection. The type of immune response triggered is largely based off how the pathogen infects:

intracellular pathogens trigger a type 1 response and extracellular pathogens trigger a type 2 response [8]. With the focus of this paper being IAV, we are most interested in exploring type 1 immunity. Briefly, a type 1 immune response is coordinated by a division of T cells referred to as T helper 1 cells, which secrete the cytokines interleukin (IL) 2 and interferon-gamma (IFN-*y*), among others, to generate a cell-mediated response that involves phagocytes, cytotoxic T cells, and cytokines protecting against an intracellular pathogen [15]. Type 1 immunity is considered protective, and is characterized by CD4+ Th1 cells, IFN-*y*, and CD8+ T cells, which protect by triggering phagocytosis [9].

T-bet and Type 1 Immunity

T-bet is a T-box transcription factor, encoded by *TBX21*, which serves as a master regulator of Th1 differentiation, but its significance extends beyond a regulator [6]. T-bet plays a role in both the innate and adaptive immune systems, being necessary for the development and function of a variety of immune cells, including natural killer cells, dendritic cells, B cells, and CD8+ T cells [16,17]. Even more interesting is T-bet's role in the creation and maintenance of memory T and B cells. Together with eomesodermin (EOMES), T-bet determines the fate of T cells; EOMES promotes memory formation while T-bet promotes terminal differentiation. [12,18-21]. EOMES is also a T-box transcription factor, but unlike T-bet, it is not greatly expressed in CD4+ T cells. Rather it is highly expressed in CD8+ T cells and has been associated with the anti-cancer properties of CD8+ cells [6]. It also plays a role in the migration of these IgG_{2a} B cells to inflammatory sites and is necessary for the survival of IgG_{2a} memory B cells [7,22].

Thus far, literature strongly suggests that help provided by the CD4 T cell subtype is responsible for the antibody switching of B cells [6]. Recent studies have also suggested that the different antibody subtypes may have varying degrees of efficacy against IAV. It is known that the different F_c regions of antibodies have different effector functions, this study sought to explore their contributions by measuring the amount of neutralizing antibodies formed against hemagglutinin. Researchers found that there were different levels of IgG_1 and IgG_{2a} produced in response to different elements of IAV, in addition to correlating a higher level of IgG_{2a} with better clearance and protection against IAV [9]. The differences in antibody efficacy is not limited to IAV, literature exploring immune responses to *Schistosoma mansoni*, *Leishmania tropica*, and cancer each found an IgG subtype to be more effective at conferring immunity [7-9]. This information brings up a very important point with regards to IAV vaccination: if different antibody isotypes have been shown to have different degrees of protection, and T-bet plays a role in antibody class switching [23], then understanding T-bet's role within an IAV setting holds great potential for strengthening the immune response and preventing the seasonal IAV epidemic. If one antibody isotype is proven more effective, vaccines can be created to induce a greater production of that isotype and thus offer greater protection.

T-bet and Antibody production

While antibodies are not the hallmark of a type 1 immune response, they are still produced in substantial amounts [15]. The most common antibody found in circulation is the IgG isotype, which is also the isotype that targets and neutralizes IAV [15]. It is unknown how T-bet affects antibody production in an influenza setting, although literature has suggested that T-bet

expression drives the generation of the IgG_{2a} isotype, while Th2 responses promote the generation of the isotype IgG [6]. Thus, it is expected that the absence of T-bet will result in decreased levels of IgG_{2a}. A follow-up question to this assumption is whether the total levels of IgG are lowered, or if another isotype is produced in greater amounts. Since T-bet appears to only induce class switching to IgG_{2a}, it is expected that the total amount of IgG will remain the same in the presence or absence of T-bet, and the IgG_1 will make up for the lack of IgG_{2a}. Recent studies have suggested that the different subtypes may have varying degrees of efficacy against IAV [24]. Exploring these differences will help us understand T-bet's role in the production of influenza-specific antibody. This information can then serve as the basis to produce a more efficient vaccine against IAV. If we can understand what isotypes offer the most protection against IAV, we can adjust vaccines to help produce the most effective immune response.

Expression of T-bet

For B cells to mount an effective immune response, they require help from T cells. There are a variety of categories that T cells offer help in, but the simplest is inducing B cell proliferation, allowing the B cells to differentiate into plasma cells and memory B cells [23-26]. T-bet is not exclusive to T cells, which leads to the question whether the differences in antibody production are attributable to T-bet expression in T cells or B cells. It cannot be assumed that expression is more important in T cells simply because of the help that they provide to B cells. Literature has suggested that T-bet expression in B cells is vital for production of IgG_{2a} [23]. Importance can only be determined by knocking out T-bet expression in either T cells or B cells and comparing the antibody production. Specifically, we would explore the amount of IgG_{2a} and,

using the results of T-bet's impact of antibody production, determine in which cell the expression of T-bet offers the most protection against IAV. As aforementioned, studies have shown that the help provided by CD4 T cells is most likely responsible for class switching, thus it is plausible to assume that T-bet expression is more significant in T cells.

Aims

In this study we seek to:

Aim 1: Explore the effect of T-bet expression on the quantity and isotype of flu-specific antibodies produced during a primary and secondary influenza infection.

Aim 2: Determine whether T-bet expression in B cell or T cells is more important for the production of flu-specific antibody.

We expect that T-bet knockout (KO) mice will have less IgG_{2a} and that T-bet expression will be more important in T cells for antibody production.

METHODS

Mice

WT or T-bet KO mice on a C57BL/6 background were used to obtain the serum samples. The mice used were between 8-12 weeks of age.

Virus

The virus variants used in this experiment include PR8 (H1N1), A/Phil (H3N2), and A/Alaska (H3N2).

Infection and Harvesting

For the IAV infections, the mice were anesthetized using isoflurane, and were then intranasally infected with 50µL virus diluted in phosphate buffered saline (PBS) at a varying priming dosage of 0.2 - 0.5 lethal dose 50% (LD_{50}). After the mice returned to their pre-infection weight, they were then challenged with a supra-lethal dose of a heterosubtypic virus in the range of $50LD_{50}$ - $200LD_{50}$.

Because most of the serum samples were taken after the heterosubtypic challenge, the blood could be harvested via cardiac puncture, where the mice are anesthetized with isoflurane and a 23-25-gauge needle is used puncture a ventricle and draw blood. For the set of Day 45 pre-heterosubtypic challenge mice, a submandibular puncture was performed, where the submandibular vein is pierced with a lancet and blood collected. To separate the serum from the plasma, the harvested blood was centrifuged at 1000-2000g for 10 minutes, and the serum was

pipetted out. All animal procedures were performed in accordance with the University of Central Florida's Animal Care and Use guidelines.

Enzyme Linked Immunosorbent Assay (ELISA)

An indirect ELISA was used to analyze the serum samples. A 96-well ELISA plate was coated first with a 1:100 dilution of PR8 IAV, accounting for a blank, conjugate blank, and negative controls, and allowed to incubate for a day. The plate was then washed three times with phosphate buffered saline with tween 20 (PBST). Serum from infected mice was then serially diluted in a flat-well plate, transferred to the ELISA plate, allowed to incubate for a day, washed three times with PBST, and then the secondary antibody was added in a 1:2000 dilution to each well and allowed to incubate for three hours. The secondary antibody used was the "anti" of the isotype of interest, which was also linked to a horseradish peroxidase (HRP) enzyme. After washing again three times with PBST, the binding in each well was determined using *o*-phenylenediamine (OPD) with acid stop (25% H_2SO_4) and the optical density was determined using a plate reader set at 492nm. To determine the endpoint, the lower limit of detection was set to be twice the mean of the conjugate blank wells.

Two different analyses were performed on the data collected, the first and more direct being the absorbance graph. The absorbance of the two groups (T-bet KO or chimeras) was compared to that of the WT throughout the dilution, and based on those values, it could be concluded whether a significant difference existed between the said group and the WT mice. If both samples reached an endpoint, set based on the conjugate blanks, an analysis of the endpoint titers was performed. Using endpoint titers offers a more specific understanding of how much

antibody was present. The main isotypes that were analyzed were IgG, IgG_1, and IgG_{2a}. There were a few analyses run on the presence of IgA, but the main focus was on the isotypes of IgG, as these are indicative of the adaptive immune response and thus is most important in the context of vaccination.

RESULTS

Serum Samples

In exploring the differences in antibody production, serum samples were acquired from the flu primed T-bet knockout (KO) mice and wild type (WT) mice. As we were interested in determining the antibody concentrations, the majority of serum samples from the infected mice were taken well-after the resolution of primary infection, a time period where we were certain there would be high levels of IgG. Both primary and heterosubtypic infected mice were used for this experiment, which allowed us to test for levels during both primary and recall conditions. This experiment looked at mice day 45 of infection and mice at day 6 and day 14 after a heterosubtypic re-challenge of IAV. A heterosubtypic challenge involves using a different subtype of a virus, in this case IAV, to illicit an immune response. The initial priming is the basic infection of the mouse with a virus, the challenge however involves giving a virus that has a different subtype.

For us to explore the difference in T-bet expression, we would need to have mice that expressed T-bet in either T cells or B cells, but not both. To achieve this a rather creative solution was used. We began by utilizing nude mice, which lack a thymus and thus T-cells, and lethally irradiated them to eliminate all hematopoietic cells and reconstituted them with either WT or T-bet KO bone marrow, and both groups were given the same WT CD4 T cells. This allows for the creation of 2mice that have T cells that express T-bet, but the B cells are either KO or WT for T-bet. This setup allowed for us to determine whether B cell expression of T-bet significantly impacts antibody production.

Assay Selection

As we were investigating levels of IAV-specific antibody isotypes in infected mice, the ELISA was selected as the most appropriate assay, considering its high sensitivity and specificity. The hemagglutination inhibition (HI) assay was considered, but ultimately the ELISA was chosen as the assay of choice because of the specificity it offered, and the nature of the experiment. The HI assay functions by antibody preventing IAV attachment to the red blood cells, and the level of inhibition is measured. The point of this experiment is to measure the amount of antibody present in the serum, not necessarily the level that is protective against IAV. When performing the ELISAs, a goal was to achieve an endpoint titer for each sample, as it would provide a more specific and accurate measurement, and thus varying dilution factors were used when creating the serial dilution to achieve an endpoint.

T-bet & Antibody Production: Total IgG

The ELISA assay using anti-IgG was run on 24 mice, and the best data from three different experiments, each repeated once to ensure accuracy, are presented here. The absorbance and titer graphs for the Day 45 pre-heterosubtypic challenge sample, primed with PR8 (H1N1), (Figure 1) compares the relative amount of antibody present in the T-bet KO mice versus WT. The absorbance and titer data are also shown for mice at Day 14 post-heterosubtypic challenge, primed with A/Alaska and challenged with PR8 (H1N1), (Figure 2).

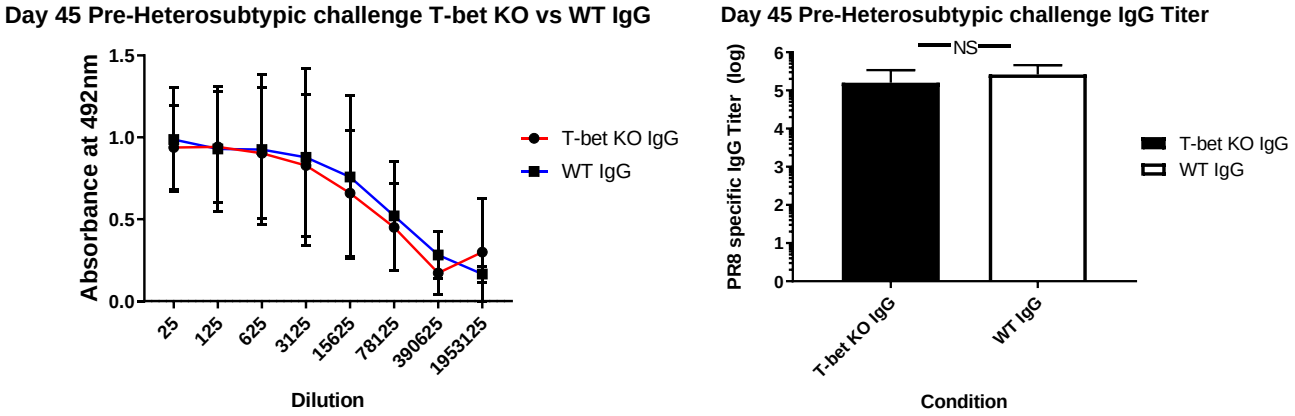

Figure 1: Absorbance (left) and titer (right) graphs for total IgG in Day 45 pre-heterosubtypic challenge mice. Six mice used in both the T-bet KO group and WT group.

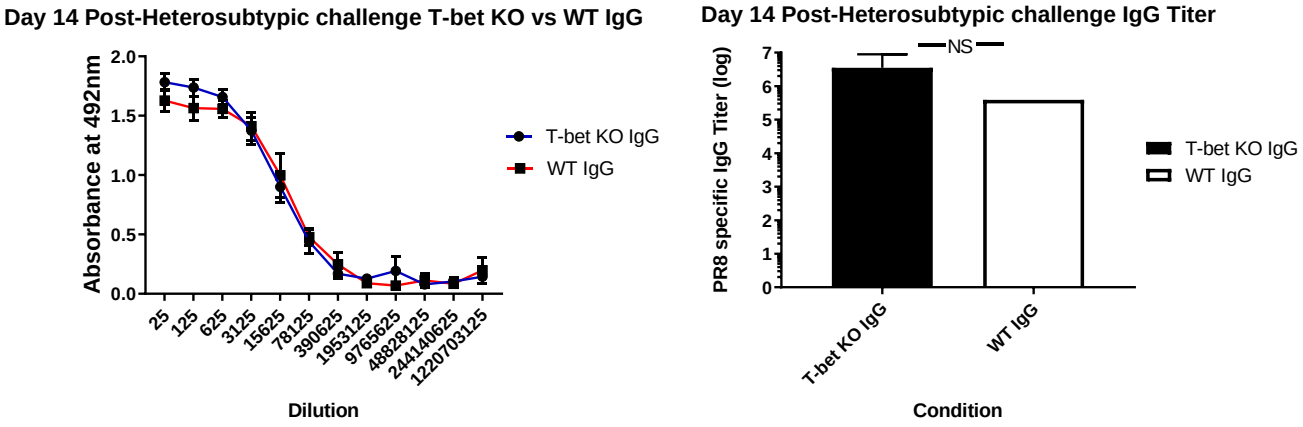

Figure 2: Absorbance (left) and titer (right) graphs for total IgG in Day 14 post-heterosubtypic challenge mice. Three mice used in both the T-bet KO group and WT group.

The absorbance data suggests that there little, if any, difference in the total amount of IgG produced by the T-bet KO or WT mice. This if further supported by the titer data, with the titers being the same or within error. Also important to note is that although there is a difference between the data for Day 45 pre-heterosubtypic challenge and Day 14 post-heterosubtypic challenge data, the trend is the same for the two groups. This shows that T-bet's control of antibody production is consistent whether it be a primary or recall condition. This also verifies

that the heterosubtypic challenge both was successful and elicits a strong adaptive immune response earlier than the primary infections, a finding that is congruent with the basic model of an immune response.

Another twist to this piece of data involves the Day 14 post-heterosubtypic challenge mice that were primed with the A/Alaska vaccination strain. Thus, this helps to draw more significance and translational application of these findings to humans.

T-bet & Antibody Production: IgG_1 & IgG_{2a}

The absorbance and titer graphs for the Day 45 pre-heterosubtypic challenge sample compare the relative amount of IgG_1 (Figure 3) and IgG_{2a} (Figure 5) present in the T-bet KO mice versus WT. Day 14 post-heterosubtypic challenge absorbance and titer data for IgG_1 and IgG_{2a} are shown in Figure 4 and Figure 6, respectively.

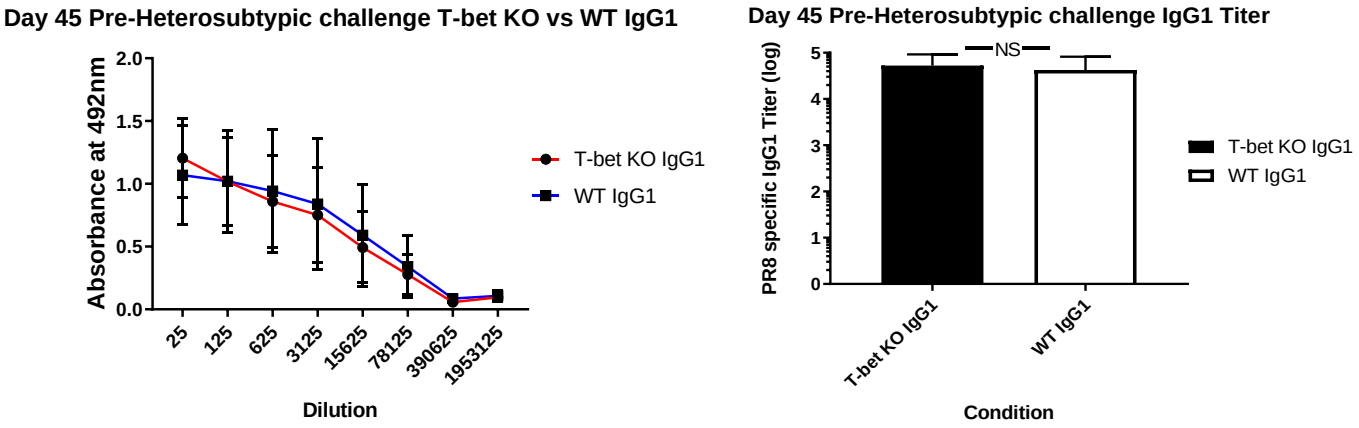

Figure 3: Absorbance (left) and titer (right) graphs for IgG_1 in Day 45 pre-heterosubtypic challenge mice. Six mice used in both the T-bet KO group and WT group.

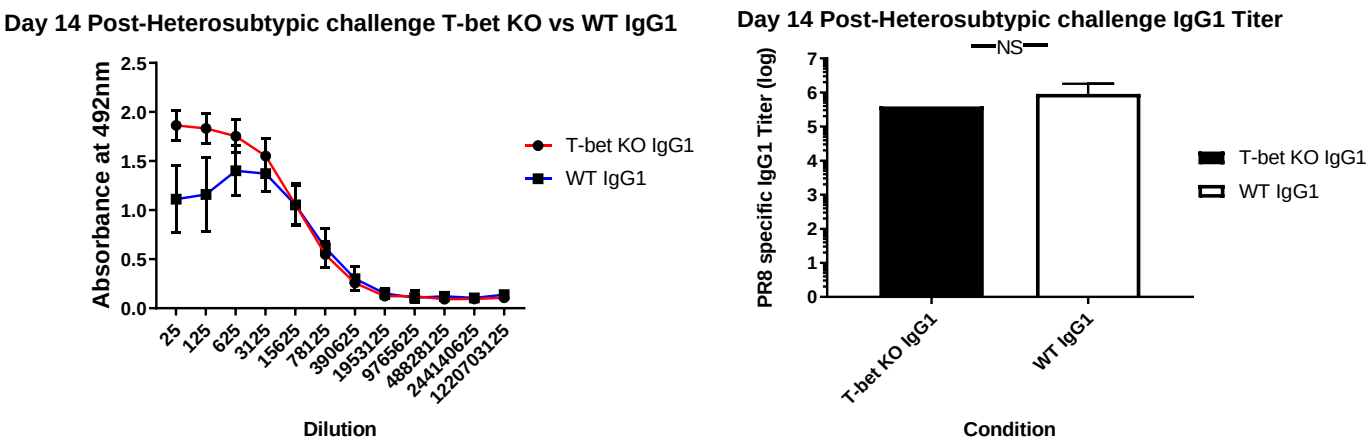

Figure 4: Absorbance (left) and titer (right) graphs for total IgG_1 in Day 14 post-heterosubtypic challenge mice. Three mice used in both the T-bet KO group and WT group.

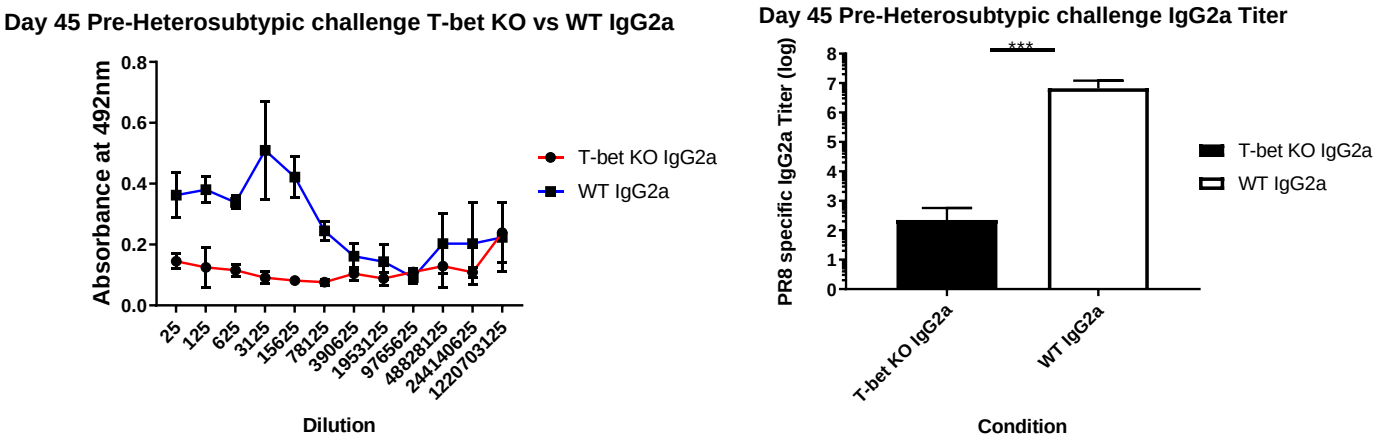

Figure 5: Absorbance (left) and titer (right) graphs for IgG_{2a} in Day 45 pre-heterosubtypic challenge mice. Six mice used in both the T-bet KO group and WT group.

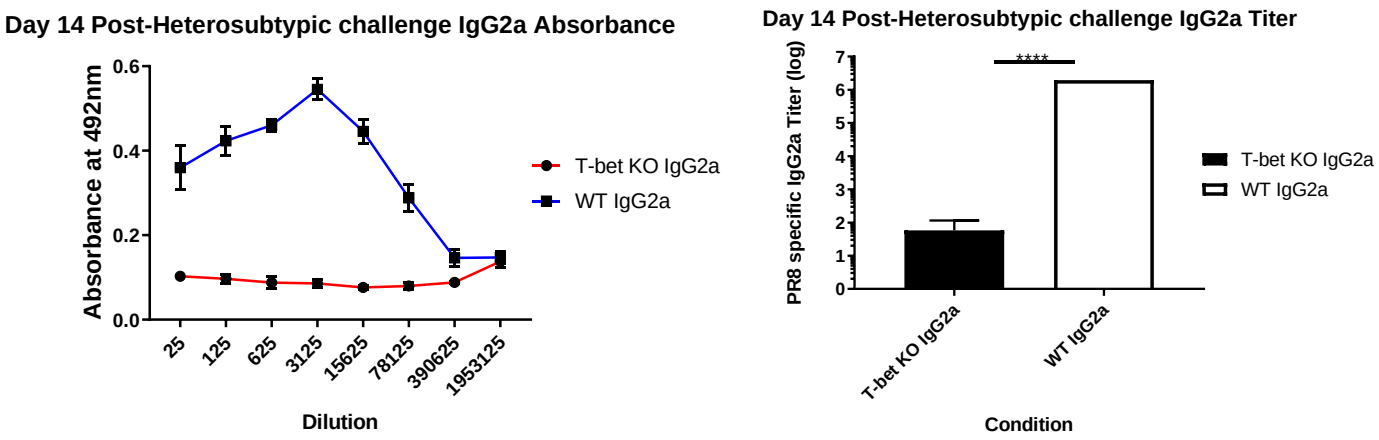

Figure 6: Absorbance (left) and titer (right) graphs for IgG_{2a} in Day 14 post-heterosubtypic challenge mice. Three mice used in both the T-bet KO group and WT group.

Staring with IgG_1, it can be seen, as with the total IgG, that there is little difference between the amount of IgG_1 present in the T-bet KO versus WT mice. However, there is a significant difference in the absorbance of IgG_{2a} in the T-bet KO and WT mice. The T-bet KO displayed a substantially lower absorbance and titer for IgG_{2a}, which is a finding consistent with literature. Since T-bet is responsible for the production and class-switching to the IgG_{2a} isotype, the knockout of the gene would be expected to have a severe impact on the amount of IgG_{2a} that is produced.

Another interesting finding from this set of data, is that despite IgG_{2a} being so low in the T-bet KO mice, the total IgG is nearly the same for the T-bet KO and the WT mice. This means that there must be some sort of compensation mechanism for the low levels of IgG_{2a}, but it cannot be the IgG_1 because those levels, again, are the same between the T-bet KO and WT mice. This suggests that there must be another subtype of IgG that is compensating for IgG_{2a}.

T-bet & Antibody Production: IgA

Another isotype explored in this experiment to a more limited extent was IgA, again looking at the absorbance and titer of Day 14 post-heterosubtypic challenge T-bet KO versus WT mice (Figure 7). As expected, there are extremely low levels of IgA in the mice, mainly because this experiment models an adaptive immune response thus IgG will be the most prominent antibody. Another reason is that IgA is mainly found as a dimer within secretions, and this experiment is looking at serum, which will contain small mounts of monomeric IgA that is secreted into the bloodstream. There is also no difference between the T-bet KO and WT mice, verifying that the presence of T-bet has no impact on the production of the IgA isotype.

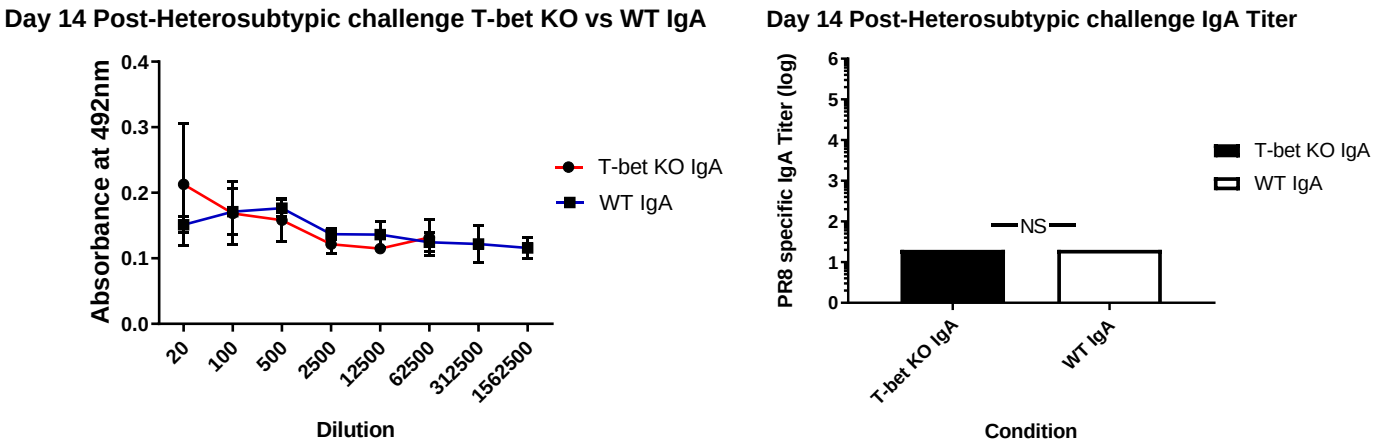

Figure 7: Absorbance (left) and titer (right) graphs for IgA in Day 14 post-heterosubtypic challenge mice. Three mice used in both the T-bet KO group and WT group.

T-bet Expression: IgG

The ELISA was also used in the second part of this experiment in exploring the significance of T-bet expression in T or B cells by using the total bone marrow (BM) chimeras. There were multiple replicates of chimeras used, but all were primed with PR8 (H1N1) and

challenged with A/phil (H3N2). The absorbance and titer data for the total IgG is shown in Figure 8.

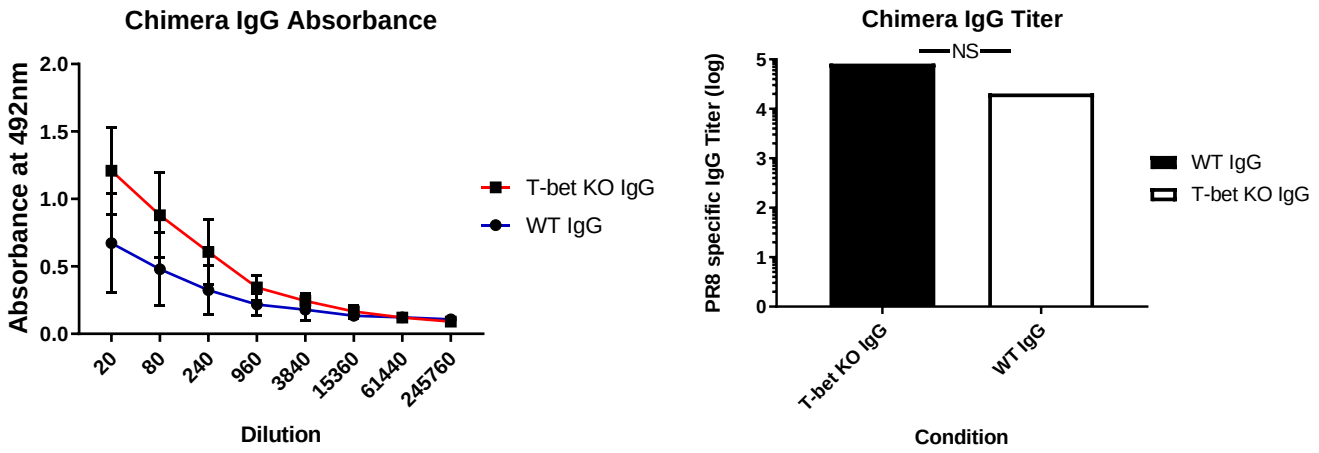

Figure 8: Absorbance (left) and titer (right) graphs for IgG in BM Chimera. Two mice used in the T-bet KO Chimera and one mouse in WT.

Similar to knocking out T-bet completely, there appears to be no significant difference between the T-bet KO and WT groups in the total amount of IgG present. This again implies that if there is a deficiency in one of the IgG isotypes, it is compensated for by another subtype. There is not much information to draw from the absorbance graph as there are overlapping error bars, and we cannot state with confidence that one group has a greater amount of antibody. These overlapping error bars were also present in the replicate, suggesting that it may not be a random error, although it could be related to the mice themselves (the WT mouse did not have a strong immune response). If the data is accurate, this suggests that the lack of T-bet expression in B cells may have actually improved the immune response in terms of antibody levels. However, the titer data, which offers a more specific quantification, does not show a significant difference. To

ensure that we are confident in the difference in total IgG, future studies should replicate this portion to confirm these trends.

T-bet Expression: IgG_1 & IgG_{2a}

We also looked at the absorbance and titers for IgG_1 (Figure 9) and IgG_{2a} (Figure 10) in the BM chimeras.

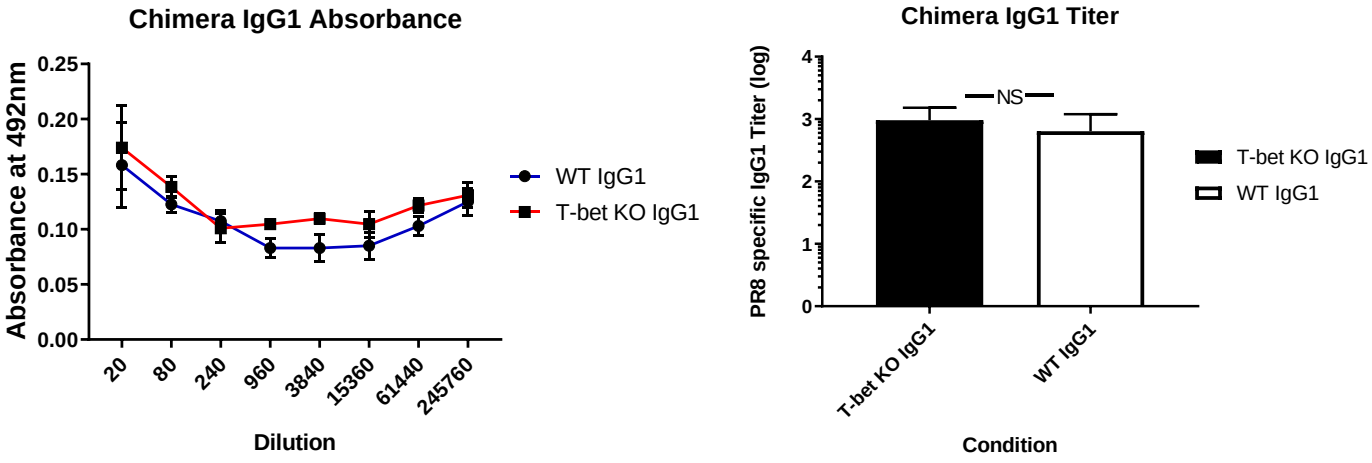

Figure 9: Absorbance (left) and titer (right) graphs for IgG_1 in BM Chimera. Two mice used in the T-bet KO Chimera and one mouse in WT.

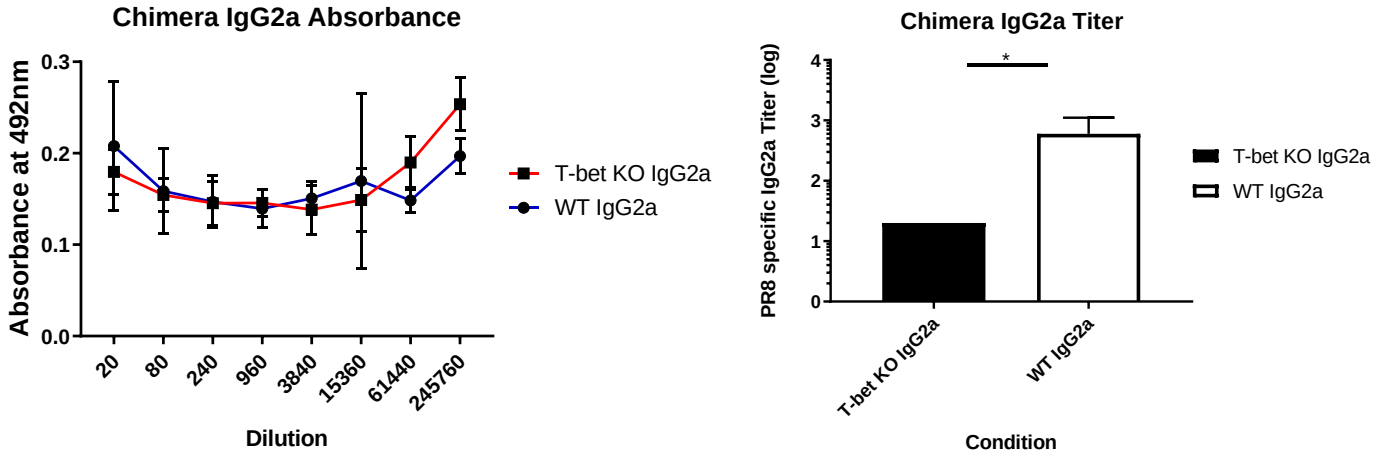

Figure 10: Absorbance (left) and titer (right) graphs for IgG_{2a} in BM Chimera. Two mice used in the T-bet KO Chimera and one mouse in WT.

The data for these two subtypes is a bit difficult to draw conclusions from. The titer data appears to show a significant difference in IgG_{2a} between the T-bet KO and WT mice, but looking at the absorbance graph, we see that both isotypes have nearly flat absorbances and have an increase in absorbance at the end of the dilution. This is not how an ELISA absorbance graph should look like, rather, the graph should have a constant slope down that is indicative of the serial dilutions that were performed on the sample. It is also worth noting that the other replicates showed similar trends, which leads us to conclude that there might have been an issue with the samples rather than a systematic or random error. Sources of these errors will be explored in the Discussion.

Even though the titer graphs do not appear skewed, those values are generated from the absorbance chart, which are unreliable to draw conclusions from. Thus this experiment would need to be repeated to obtain any accurate conclusions.

DISCUSSION

T-bet is a transcription factor that plays a role in both innate and adaptive immunity, in addition to being a master regulator of type 1 immunity. T-bet has been shown to affect the class switching of antibodies, to IgG_{2a} specifically, which is important in the setting of IAV as these flu specific antibodies are what help the immune system mount a strong and successful immune response against IAV. In exploring the impact of T-bet on the production of antibody isotypes in a mouse model of IAV, we discovered that the levels of IgG_{2a} were indeed significantly lower in the T-bet KO versus the WT, which concurs with literature. Also interesting is that the levels of total IgG and IgG_1 are very nearly the same between the T-bet KO and WT groups. This means that although the T-bet KO mice might be deficient in the IgG_{2a} isotype, they are not deficient in the total amount of IgG. This leads to the question of which antibody isotype is making up for the deficiency in IgG_{2a}, and the answer is not IgG_1, because those levels are also the same between both groups, the answer might be within another isotype of IgG not explored in this study, such as IgG_3 or IgG_{2b}, and can be the focus of future research: exploring what antibody isotype is higher in T-bet KO and whether it is that isotype that T-bet switches to IgG_{2a} or whether it is another factor that compensates in the absence of T-bet.

Another important finding of this study is that the heterosubtypic challenge was successful in that it produced a stronger immune response than the primary infection state, but also showed the same trends in antibody levels in the T-bet KO mice. In all the experiments and replicates, the Day 45 pre-heterosubtypic challenge mice showed similar absorbances and titers to that of the Day 6 and Day 14 post-heterosubtypic challenge mice.

The experiment from exploring the possible differences in the expression of T-bet did not turn out as well as we had hoped. The absorbance graphs appeared to be flat, indicating that the ELISA was not performed properly. This error might be due to the samples themselves, however there are multiple points in the protocol where an error could have been made. For example, the dilution may have not been made properly, or there could have been a mistake made when transferring from the dilution plate to the ELISA plate. There also may have been an issue with the reagents, in that they were left out too long before performing the assay. Another possible source of error could be that the plates were allowed to develop for too long, so the differences between the dilutions were lost. Thus, this experiment should be repeated taking the abovementioned sources of error into consideration. The future experiment should also explore the other side of T-bet dependence by eliminating T-bet in all B cells and making expression of T-bet in T cells the independent variable.

Regarding the assay used for this study, the ELISA has multiple pros and cons. The ELISA does offer a high degree of specificity and sensitivity. However, the ELISA is limited in that it is dependent on the amount of antibody and the interactions that form between them. If there is a small amount of antibody or the proteins dry up, the results will be flawed. Another issue comes from the dilutions made, if the dilution was too wide, the actual endpoint might fall between two dilutions and thus give a false number for the endpoint. Another potential source of error seen with this experiment was an increase in absorbance at the end of the plate where the dilution was the highest. This was a consistent error in the ELISA data, but it does not severely impact the data, as the endpoint titer was often achieved before the absorbance values began to increase.

This study was meant to serve as an introduction to T-bet and an immune response against IAV. This study demonstrated that T-bet does impact the levels of IgG_{2a} but there was no clear conclusion of where the expression of T-bet is most significant in the antibody response against IAV. Pairing this information with the literature that asserts that IgG_{2a} is important for protection against IAV [12], in the context of vaccination, this means we should be exploring vaccines that illicit a greater production of IgG_{2a}, and T-bet could be a vector to achieve this. Now that we understand the role T-bet plays with antibody production in an IAV setting, future studies should explore how manipulating expression of T-bet can increase the amount of class switching to IgG_{2a}. This would allow us to produce a more effective vaccine against IAV. These findings are not limited to IAV however, if we understand what antibody subtypes offer the most protection against a pathogen, cancer, or even autoimmune disease, the subtypes can be manipulated through T-bet, or another transcription factor, to help the body mount the most effective and protective response possible.

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